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Bone Marrow Stromal Cells (BMSCs) in Bone Engineering: Limitations and Recent Advances

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Abstract—Bone marrow stromal cells (BMSCs) have been isolated for the first time by Friedenstein *et al.* and since then have been considered the progenitor cells for the skeletal tissues. Indeed BMSCs are clonogenic, fibroblastic in shape, and can differentiate along multiple lineages such as osteoblasts, chondrocytes, adipocytes, and hematopoiesis-supportive stroma. When implanted *in vivo* on a three-dimensional bioceramic scaffold into immunocompromised mice, BMSCs form bone and hematopoiesis-supportive stroma. The ease of harvest from a donor bone marrow together with the ability to form bone *in vivo* make BMSCs ideal for clinical applications. Thus, *ex vivo* expanded BMSCs have been employed, first in large animal models, then in human clinical trials, to repair large bone segmental defects. Further investigation of the expanded BMSC population led to the observation that *in vitro* expansion appears a limiting passage: cells tend to senesce and lose their multidifferentiation potential with time in culture. To overcome these limitations, two approaches have been proposed: (1) identification of the appropriate culture conditions to prevent senescence by possibly selecting a subpopulation with stem cell characteristics, and (2) engineering of the cells by transfection with the telomerase gene to prevent cells from telomere shortening and consequent aging.

Keywords—Bone marrow, Stem cells, Aging, Telomerase.

BONE MARROW STROMAL CELLS (BMSCs)

In the adult life of the organism the skeleton is completely formed, but continues to undergo constant remodeling to prevent accumulation of microdamages and allow for fracture healing. This suggests that a population exists which retains stem/progenitor cell properties and remains somewhat quiescent throughout the entire life of the organism, intervening only when needed to form new bone.

Friedenstein *et al.* were the first to identify in the adult bone marrow a cell population with strong osteogenic potential.[16,17] They harvested whole bone marrow, gently kneaded it, and easily obtained a marrow single-cell suspension. This single-cell suspension was plated and the culture dish repeatedly washed to remove nonadherent hematopoietic cells. After a few days in culture Friedenstein and associates observed cells attached to the plastic that appeared fibroblastic in shape and were actively proliferating.

When plated as described above at low cell density, BMSCs form colonies and are therefore defined as Colony Forming Units—Fibroblasts (CFU-Fs), indicating that each colony derives from a single proliferating progenitor.[32] Interestingly, when BMSCs are implanted *in vivo* in either closed systems (diffusion chambers) or open systems (under the renal capsule or subcutaneosly) they form bone and hematopoiesis supportive stroma reconstituting a complete bone/bone marrow organ.[7,18,26,28,31]

Additional studies conducted by several groups not only confirmed the bone-forming capacity of this cell population and the role of BMSCs as osteo progenitors, but also showed that BMSCs can undergo differentiation towards multiple mesenchymal lineages such as chondrocytes and adipocytes.[1,5,17,32,36,42]

More recent work indicates that BMSCs, also indicated as MAPCs (Multipotent Adult Progenitor Cells), MSCs (Mesenchymal Stem Cells), BMSSCs (Bone Marrow Stromal Stem Cells), and MPCs (Mesodermal Progenitor Cells), if appropriately induced can also undergo "unorthodox" differentiation[8] where the cell undergoes a different pathway from the one of the tissues of origin. Sanchez-Ramoz demonstrated that BMSCs could differentiate into neurons,[41] whereas Ferrari *et al.*[15] have very nicely shown that BMSCs, can actively participate in the repair of skeletal muscle tissue.[15]

In this respect BMSCs represent an important example of adult stem/progenitor cells and as such an interesting source of cells for therapeutic applications. The question arises though if this population is homogenous, or whether it comprises a mixture of cells committed to various lineages of differentiation, where only a subpopulation represents the true stem cell pool.

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Thus, the heterogeneous nature of the population is immediately evident by the observation of BMSC single colonies.[32] Colonies appear different in shape and size, reflecting differences in proliferation and growth rate among the CFU-Fs. After longer times in culture, phenotypic differences are more evident. Few colonies express early osteogenic markers such as alkaline phosphatase (ALP) and few stain positive for oil red O (adipogenic commitment) or deposit minerals.[26]

A more definitive proof that BMSCs are a mixed pool of cells at various stages of commitment is given by the evaluation of the mature phenotypes of the cells derived from single-cell clones.[32] Muraglia and coworkers demonstrated that clonogenic cells from a BMSC population had different degrees of differentiation potential.[30] Few CFU-Fs were tripotential in that they could differentiate *in vitro* into osteoblasts, chondrocytes, and adipocytes, others were only bipotential differentiating only towards a chondro-osteogenic phenotype, and a third group comprised only osteogenic clones. Interestingly it appeared that with time in culture the differentiation potential was somewhat reduced and tripotential clones progressively lost the adipogenic, the chondrogenic, and lastly the osteogenic potential suggesting a preferential commitment of the progenitors towards the osteogenic phenotype.[30]

Further proof of the heterogeneity of the BMSC population came from experiments conducted by Kuznetsov and coworkers, who demonstrated that only 60% of the single-colony-derived strains implanted subcutaneously into immunocompromised mice on a bioceramic scaffold gave rise to bone.[26] Among these only 65% differentiated towards both bone- and hematopoiesis-supportive stroma.

This and other evidence indicates that bone marrow stromal cells represent a very heterogeneous population comprising cells at different stages of commitment.

All these studies though were not successful in determining the nature of the heterogeneity of the BMSC population. Differences observed with regard to their morphology and their bone-forming ability, as well as the multipotential of the population cannot be ascribed with certainty to the intrinsic nature of the population instead of the different *in vitro* culture conditions. The lack of specific markers for the bone marrow stromal cell makes the evaluation of the nature of heterogeneity still difficult. Studies are being performed to establish molecular markers that will enable to distinguish among subpopulation and/or different stages of commitment.

BMSCs AND BONE RECONSTRUCTION

Since the first evidence of the ability of BMSCs to form bone *in vivo* great interest has been shifted towards a possible application of these cells in the reconstruction of bone.

Following extensive experimentation on small animal models of bone formation and repair, Bruder *et al.* provided the first proof of the possible application of BMSCs for the reconstruction of long segmental defects in larger animals.[11] Similar studies were conducted by Kon *et al.*, who created a segmental defect in the long bones of sheep and designed a ceramic cylinder of porous hydroxyapatite to fill the gap. The cylinder was implanted alone or loaded with *ex vivo* expanded autologous bone marrow stromal cells. Bone reconstruction and formation was evaluated 2 months after surgery by histology and radiography (including microradiography).

Analysis of the implant revealed that bone formed in both implants, supplemented and not supplemented with cells, but that the rate of bone formation increased considerably in implants loaded with cells. Furthermore they reported that recovered bioceramic loaded with BMSCs showed a bone with higher stiffness and more importantly bone formed on the borders of the ceramic as well as in the internal pores.[24]

Similar results were also obtained by Petite *et al.*, who repaired critical size bone segmental defects in sheep by implantation of a coral-based scaffold alone or together with *ex vivo* expanded BMSCs.[34] They observed that only in the coral implants supplemented with BMSC, bone formed both in cortical and medullar areas and in three out of seven animals implanted, clinical union of the segmental defects was obtained.

The encouraging results obtained with implants of BMSCs cultured *ex vivo*, first in the nude mouse model, then in larger animals, led to initiate human clinical trials on those patients where all other traditional therapeutic approaches had proven unsuccessful.

The first clinical report appeared in the *New England Journal of Medicine* where an interdisciplinary effort was undertaken to treat three patients with various segmental defects (4-cm bone segment loss in the right tibia, 4 cm in the right ulna, and 7 cm in the right humerus) using the above-mentioned cell-based therapeutic approach.[38] Whole bone marrow was harvested from each patient and BMSCs were *ex vivo* expanded and loaded on a three-dimensional scaffold of the shape and size of the missing bone fragment. External fixation was provided for stability and removed after 6.5, 6, and 13 months, respectively. All three patients presented a repair of the fracture site: all the implants showed good integration of the newly formed bone and abundant callus formation. Recovery was obtained at earlier times than those employed by the standard therapeutic approaches (bone flaps).

EX VIVO EXPANSION: A CRITICAL STEP

For clinical applications it is of critical importance that the cells to be used have a high regenerative potential, in order to maintain a tissue or a function over a lifetime. The use of BMSCs for the reconstruction of bone segmental defects requires extensive expansion *in vitro* of the cell population. In this respect, several studies have been conducted to

evaluate how the proliferation rate and the differentiation potential of bone marrow stromal cells are affected during the *ex vivo* expansion phase.

Bruder *et al.* reported that BMSCs isolated from fresh bone marrow aspirates and extensively expanded *in vitro* undergo senescence and a change in shape and morphology after about 38 population doubling. Expression of ALP and an *in vitro* assay of bone nodule formation appeared not to be affected by this replicative senescence indicating that although unable to replicate these cells maintain the ability to differentiate towards the osteogenic phenotype.[10] Banfi *et al.* reported similar conclusions, showing that BMSCs derived from three different donors and passaged in culture undergo a progressive decrease of the proliferation rate until the 22nd–23rd cell doubling, when a drastic drop in their proliferation capacity was observed.[4] In this study, though bone formation was evaluated in the nude mouse assay at different culture times and it was evident that even the osteogenic potential of the *ex vivo* expanded cell population was reduced when compared to that of fresh bone marrow. Similar results were also obtained when differentiation was evaluated in cell populations derived from a single-cell.[30] Thus, in all cases the adipogenic differentiation potential was the first to be lost. Overall, these data indicate that *ex vivo* expansion leads to a progressive decrease of proliferation and loss of the multilineage differentiation potential.

Several studies have been conducted to improve the culture conditions and optimize the expansion protocol.[19,25,27,35,39] In particular the addition of FGF2 to the culture media during the initial cell plating and during the cell expansion phase increments the cell proliferation rate, maintains the cell differentiation potential, and increases the amount of bone formed *in vivo* possibly selecting a more primitive subpopulation.[4,6,29]

In normal somatic adult cells each cell division causes progressive shortening of telomeres eventually resulting in an arrest of cell growth and proliferation. In fact, only true stem cells or malignant cells are able to self-renew and proliferate extensively possibly in view of the fact that they express high amounts of the enzyme telomerase. Telomerase is an enzyme that prevents telomere shortening by extending their length at the end of the chromosomes, therefore preventing the cells from progressively aging. BMSCs, as most somatic cells, do not express telomerase and as a result subsequent cell passages in culture are characterized by progressive shortening of the telomeres and consequent cell aging.[3,6]

From all these studies it must be concluded that BMSCs do not replicate indefinitely *in vitro* and undergo senescence. The addition of FGF2 to the culture even though selecting a subpopulation of earlier progenitors among the total BMSC population can only delay culture senescence.[29] Two are the possible explanations, either within the BMSC population resides indeed a stem cell compartment, but culture conditions or the environment so far have not been permissive to support its isolation and expansion or the BMSC is not a true stem cell.

If the first hypothesis is correct, to overcome the limitations of BMSC expansion, one should develop optimal culture conditions to select/isolate a stem cell subpopulation in the marrow that could self-renew indefinitely and keep unaltered its multi-differentiation capability even after prolonged culture. A second approach could aim at engineering the cells by transfection with the human Telomerase Reverse Transcriptase (hTERT) to overcome telomere shortening in culture and senescence *in vitro*.

WHERE ARE WE TODAY?

A great contribution in this respect has come from Reyes *et al.*[40] From multinucleated cells of human bone marrow, they were able to isolate a cell population, indicated as MPCs, with undoubtedly stem cell characteristics.

Reyes and coworkers harvested bone marrow from 30 healthy donors and depleted the obtained suspension from CD45+ glycoforin A+ cells, postulating that mesenchymal progenitors had to be different from hematopoietic ones. Cells were then plated on fibronectin-coated dishes and only 0.02–0.08% of the CD45− GlyA− population attached and formed proliferating cell clusters. MPCs were then cultured in the presence of selected growth factors: 10-ng/ml EGF and 10-ng/ml PDGF and kept at a constant low cell density of no more than 2 and $8 \times 10^3/cm^2$. Furthermore, expansion was conducted in the presence of a low concentration of fetal calf serum (2%) for the entire time in culture.

Only MPCs maintained in the above-mentioned culture conditions proliferate up to 50 cell doublings without obvious senescence maintaining their differentiation potential. Telomere length remained unchanged during the entire cell life span indicating that the cells did not undergo senescence under these conditions.

In addition, not only MPCs could undergo differentiation towards the osteo-chondro-adipogenic lineage but they were also able to differentiate towards cells of the visceral mesoderm: endothelium and muscle cells, as evidenced by the expression of von Willebrand Factor and myosin, respectively.

The above protocol could be the missing key for an optimal utilization of the bone marrow stromal cell population in the clinic. Once other laboratories would have reproduced the experiments, the possibility to isolate a subpopulation in the marrow that, even though very limited, can proliferate extensively and yet differentiate will open enormously the applicability of BMSCs.

In addition or in alternative to this approach, two laboratories have independently proposed engineering of BMSC by transducing the cells with the hTERT cDNA to optimize their life span and differentiation potential.[43,44] It has been shown[44] that *in vitro* culturing of human osteoblastic

cells determines senescence and eventually growth arrest. Furthermore evaluation of gene expression profiles of osteoblastic cells in culture appears altered with time in culture. One of the possible causes of cellular senescence has been ascribed to telomere shortening.[20] The telomerase gene when present determines the maintenance of the telomere length, which shortens otherwise at each cell division. Telomerase (hTERT) is absent or present at very low levels in most somatic cells, while it seems to be highly expressed by proliferating germ line cells, hematopoietic stem cells, and various types of cancer cells. BMSCs do not express telomerase (hTERT) and this might contribute to the progressive senescence of this population in culture.[9,22,23]

Both groups were able to demonstrate that hTERT-transduced BMSCs increased notably their proliferation rate reaching up to 80 population doublings and telomere length remained constant up to the population doubling tested. BMSC-transduced cells keep a normal kariotype,[44] never grow in soft agar, or develop tumors when injected subcutaneously into immunocompromised mice.[43]

Bone formation *in vitro* was evaluated by Shi *et al.* looking for the expression of ALP, an early marker of osteogenesis and for mineral deposits by Von Kossa staining. hTERT transfected and *in vitro* expanded cells, after only 2 weeks of osteoinduction, expressed considerably more ALP than control cells, and after 4 weeks produced considerably more calcified matrix than not transfected control cells.[43]

In vivo, transduced BMSCs form bone when implanted within a hydroxyapatite/tricalcium phosphate scaffold subcutaneously into immunocompromised mice. Thus, in an *in vivo* bone formation assay, culture expanded hTERT-BMSC formed up to 10 times more bone than not transfected control cells.[43]

CONCLUSIONS

Bone Marrow Stromal Cells constitute a very heterogeneous cell population that resides in the bone marrow and can be isolated and expanded manifold *in vitro*. BMSCs have been shown to differentiate upon appropriate induction into osteoblasts, chondrocytes, adipocytes, and hematopoiesis-supportive stroma and thus have been proposed as progenitor cells for most tissues of the adult skeleton.

In the recent past though BMSCs have been shown to contain "stem/progenitor cells" self-renewing and able to differentiate in multiple lineages including tissues other than the one of origin such as neurons, hepatocytes, and skeletal muscle cells.[15,33,40,41]

BMSCs are an ideal candidate for therapeutic applications since they are relatively easy to harvest, easily expandable *in vitro*, and can be isolated from adult bone marrow still retaining their differentiation potential.[32,37] BMSCs have been used to repair damaged skeletal tissue or large bone segmental defects.[8,24,34,38] In the recent past though other sources of bone progenitor cells have been identified.[2,12–14,45,47] Studies are on their way to evaluate possible employment of these populations for engineering of the skeleton.[46] These studies, although very promising, still need to be further extended. Vacanti *et al.* have recently reported the employment of periosteal-derived stem cells in conjunction with coral scaffold to reconstruct an avulsed phalanx.[46] Successful use of BMSCs and of all these different sources though is still linked to the *ex vivo* expansion phase, critical to provide the amount of cells required for bone reconstruction when the defect is extensive. Several studies in the recent past have thus focused on the characterization of the BMSC population in culture. Data available today indicate that cultured BMSCs progressively lose their proliferation potential.[3,4] Cells reduce their growth rate with time in culture, change from spindle shape to a flattened, large morphology, and show a decrease of the telomere length, all signs of a population aging. Prolonged *ex vivo* expansion also affects BMSC differentiation potential. Great efforts have been undertaken to overcome this problem and two approaches have been developed: (1) selection of appropriate culture conditions to isolate a subpopulation within the bone marrow that retain "stem cell" properties of self-renewal and multidifferentiation potential over time and (2) transfection of BMSCs with the hTERT cDNA to prevent telomere shortening and consequent culture senescence.

To date both approaches appear promising in the field of bone tissue engineering further studies are though required to allow their safe and convenient application in the clinic. The isolation and selection of a stem cell population, although very promising, still has some limitations because of the time needed to isolate the stem cells (only 0.02%), to plate, and to expand them to reach the amount needed. On the contrary, hTERT-transfected cells, being an engineered cell population that appears to proliferate indefinitely, need to be overly controlled for tumor transformation and overgrowth complications when implanted in humans. In addition, the time required to engineer the cells need to be taken into account considering that each patient needs to have its own bone marrow stromal cells engineered in order to prevent body reaction to the transplant.

Possibly hTERT-transfected BMSCs will find a useful application in long-term bone marrow cultures for the support of *ex vivo* expansion of the hematopoietic compartment. Hematopoietic progenitors crucial for remission of severe hematologic disorders are thought to be highly abundant in the cord blood but still not sufficient for transplantation of an adult patient. As Kawano *et al.* nicely show hTERT BMSCs increase drastically the amount of hematopoietic CD34+ cells from cord blood obtained after *ex vivo* expansion.[21] This approach might improve considerably the transplant protocols opening great perspective into the hematopoietic stem cell transplantation protocols.

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